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Characterization of the in vitro kinetic interaction of chlorpyrifos-oxon with rat salivary cholinesterase: A potential biomonitoring matrix

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Abstract

The primary mechanism of action for organophosphorus (OP) insecticides such as chlorpyrifos (CPF) involves the inhibition of acetylcholinesterase (AChE) by their active oxon metabolites resulting in a wide range of neurotoxic effects. These oxons also inhibit other cholinesterases (ChE) such as butyrylcholinesterase (BuChE), which represents a detoxification mechanism and a potential biomarker for OP insecticide exposure/response. Salivary biomonitoring has recently been explored as a practical method for examination of chemical exposure, however, there are few studies exploring the use of saliva for OP insecticides. To evaluate the use of salivary ChE as a biological monitor for OP insecticide exposure, a modified Ellman assay in conjunction with a pharmacodynamic model was used to characterize salivary ChE in adult male Sprague–Dawley rats. Comparison of rat saliva, brain, and plasma ChE activity in the presence of selective inhibitors of AChE and BuChE (BW284C51 and iso-OMPA, respectively) with different ChE substrates indicated that rat salivary ChE activity is primarily associated with BuChE ($>95\%$). Further characterization of rat salivary BuChE kinetics yielded an average total BuChE active site concentration of 1.20 ± 0.13 fmol ml^{-1} saliva, an average reactivation rate constant (Kr) of 0.070 ± 0.008 h^{-1}, and an inhibitory rate constant (Ki) of ~ 9 nM^{-1} h^{-1}. The pharmacodynamic model successfully described the in vitro BuChE activity profile as well as the kinetic parameters. These results support the potential utility of saliva as a biomonitoring matrix for evaluating occupational and environmental exposure to CPF and other OP insecticides.

Keywords: Saliva; Cholinesterase; Organophosphate insecticide; Chlorpyrifos; Chloropyrifos-oxon; Biomonitoring

1. Introduction

Organophosphorus (OP) insecticides like chlorpyrifos (CPF) are used extensively in the agricultural industry and certain residential applications. The primary mechanism of action and the most

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acutely life-threatening effect of the OP insecticides results from the inhibition of acetylcholinesterase (AChE) by their active oxon metabolites with subsequent accumulation of acetylcholine within the cholinergic synapses resulting in a wide range of neurotoxic effects (Mileson et al., 1998). The oxons are more potent inhibitors of AChE than are the parent compounds by about three orders of magnitude (Chambers and Chambers, 1989; Forsyth and Chambers, 1989). The toxicity of a common OP insecticide CPF involves CYP450-mediated oxidative desulfuration to CPF-oxon (Sultatos and Murphy, 1983; Chambers et al., 1994; Amitai et al., 1998). In addition, CYP450s also mediate the detoxification of CPF via dearylation, producing diethylphosphate and trichloropyridinol (Ma and Chambers, 1994, 1995). The balance between desulfuration and dearylation results in very different levels of AChE inhibition (Timchalk, 2001; Timchalk et al., 2002a). Several studies have indicated that the oxons are potent inhibitors of other non-target B-esterases such as butyrylcholinesterase (BuChE) and carboxylesterases (CaE: Aldridge, 1953; Mendoza et al., 1971; Fonnum and Sterri, 1981; Costa et al., 1999). Inhibition of these esterases is considered a potential protective mechanism against AChE inhibition since BuChE and CaE stoichiometrically detoxify some of the oxon and prevent that fraction from inhibiting AChE (Junge and Krisch, 1975; Fonnum et al., 1985; Maxwell et al., 1987; Chambers et al., 1990; Chanda et al., 1997; Yang and Dettbarn, 1998).

Globally, there are approximately 3 million episodes of OP insecticide poisoning a year, resulting in nearly 200 000 deaths (Haywood and Karalliedde, 2000). In addition, the general public are exposed to low levels of OP insecticide through ingestion of residues on foods and contaminated water supplies as well as through inhalation and dermal contact. Due to the potential for significant acute toxicity, there is a need to accurately quantify OP insecticide exposure. In this regard, biological monitoring is a critical tool for evaluating exposure to a wide variety of chemical agents including OP insecticides (Chester, 1993; Woollen, 1993).

Organophosphate insecticide biomontoring has been primarily focused on the assessment of cholinesterases (ChE) activity in blood and or the quantification of metabolites in urine (Peoples and Knaak, 1982; Chester, 1993). Non-invasive methods have been advocated for quantifying the pharmacokinetics and bioavailability of drugs and xenobiotics (Graham, 1982). The use of saliva has been suggested as an ideal body fluid that could be substituted for blood in biomonitoring (Pichini et al., 1996). Saliva, as a biological fluid, is simple to obtain and does not require medical professionals or invasive collection procedures (Lu et al., 1997). Saliva has been utilized successfully for monitoring therapeutic drugs, hormones and enzymes (Nakamura and Slots, 1983; Vinning and McGinley, 1987; Drobitch and Svensson, 1992; Hansen et al., 1992; Nagler et al., 2001); however, very limited information about rat or human salivary ChE enzyme activity has been reported in the literature. Borzelleca and Skalsky (1980) noted that in rats exposed to carbaryl; salivary ChE inhibition kinetics were consistent with the plasma and red blood cell (RBC) ChE inhibition. It was also demonstrated that the saliva carbaryl metabolite concentrations correlated with the metabolite blood concentrations. Collectively this suggests that both the saliva chemical concentration and the ChE activity can be used to predict the chemical concentration and ChE activity in the blood.

Characterization of AChE and BuChE in various mammalian tissues is usually distinguished on the basis of substrate specificity (Augustinsson, 1948). AChE rapidly hydrolyzes acetylcholine and shows no activity towards butyrylcholine, which is a preferred substrate for BuChE. Selective inhibitors have been identified for ChE enzymes, BW284C51 and GD-42 for AChE and iso-OMPA and ethopropazine for BuChE (O'Brien, 1960; Silver, 1974). The current study was undertaken to characterize the total salivary ChE activity and estimate the kinetic parameters of in vitro interaction of CPF-oxon with adult male Sprague–Dawley rat salivary ChE.

2. Materials and methods

2.1. Chemicals

Chloropyrifos-oxon was purchased from Chem Service Inc. (West Chester, PA), *S*-butyrylthiocholine chloride (BTC), 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB), acetylthiocholine chloride (ATC), 1,5-bis (4-allyldimethylammonium-phenyl) pentan-3-one dibromide (BW284C51) and tetraisopropylpyrophosphoramide (iso-OMPA) were purchased from Sigma Chemical Company (St Louis, MO), ketamine hydrochloride was purchased from Abbott Laboratories (Abbott Park, IL) and xylazine was purchased from Wildlife Pharmaceuticals Inc. (Fort Collins, CO). The remaining chemicals used in this study were either reagent grade or better and were purchased from Sigma Chemical Company.

2.2. Animals

Adult male Sprague–Dawley rats (300–350 g) were purchased from Charles River Laboratories Inc. (Raleigh, NC). The animals were housed in solid-bottom cages with hardwood chips under standard laboratory conditions and given free access to water and food (PMI 5002, Certified Rodent Diet). All procedures involving animals were in accordance with protocols established in the NIH/NRC *Guide and Use of Laboratory Animals* and were reviewed by the Institutional Animal Care and Use Committee of Battelle, Pacific Northwest Division.

2.3. Saliva, blood and brain preparation

Rats were anesthetized using an ip injection of ketamine:xylazine (87:13 mg kg^{-1}) then administered pilocarpine (ip; 1 mg kg^{-1}) to induce salivation 10–15 min prior to starting saliva collection. Pilocarpine is a cholinomimetic agent that binds to and activates cholinergic receptors (mainly muscarinic) without affecting ChE. It should be noted that the use of pilocarpine did not appear to affect ChE activity as evidenced by similar activity measured in brain and plasma in rats with and without pilocarpine administration. Saliva ($\sim$0.5–3 ml) was collected over a 30 min period using glass capillary tubes. Characterization of total salivary ChE activity was determined using individual rat samples ($n = 3$ rats per experiment) while in vitro salivary ChE kinetics were studied using saliva samples pooled from four animals. Upon completion of saliva collection, blood was drawn from the posterior vena cava using a heparinized syringe and centrifuged for 10 min ($2000 \times g$). The hematocrit was determined and the plasma was separated and diluted in 9 volumes of 0.1 M phosphate buffer (pH 7.4). Following exsanguination, rats were decapitated and each rat brain was immediately removed, rinsed in ice cold phosphate buffer, weighed, and homogenized in 9 volumes of buffer using a polytron homogenizer. All samples were stored at -80 °C until the time of the experiments.

2.4. Determination of the total ChE enzyme activity

Total sample ChE activity was determined from the capacity of the enzyme to hydrolyze ATC, since ATC is a good substrate for both AChE and BuChE. BuChE activity was determined from the capacity of the enzyme to hydrolyze ATC in samples incubated with BW284C51 or by the capacity to hydrolyze BTC in the absence of ChE inhibitors, since only BuChE has the capacity to hydrolyze BTC (Mikalsen et al., 1986; Dupree and Bigbee, 1994; Lassiter et al., 1998; Chuiko, 2000). AChE activity was determined from the capacity of the enzyme to hydrolyze ATC in samples incubated with iso-OMPA (Maxwell et al., 1987; Silver, 1974). There were no previously reported data concerning the use of ChE inhibitors for rat salivary ChE activity characterization, therefore, the optimal concentration of BW284C51 and iso-OMPA was determined by incubation of saliva samples with several inhibitor concentrations for different time periods (data not shown).

The optimal BTC substrate concentration was determined using a BTC concentration range between 0.1 and 10 mM and the non-enzymatic substrate hydrolysis was studied by inclusion of a parallel buffer sample containing no homogenate. A BTC concentration of 0.4 mM was determined

as an optimal substrate concentration for all assays.

The brain, plasma and saliva samples were thawed at room temperature and aliquots of the brain homogenate were diluted in 6 volumes of phosphate buffer, while the plasma was diluted in 10 or 4 volumes of buffer to determine AChE and BuChE activity, respectively. Preliminary studies indicated that the saliva samples could only be diluted up to 6 volumes of buffer in order to achieve reasonable salivary ChE activity. To determine the total ChE activity and the specific ChE associated with that activity, 200-μl aliquots of the diluted samples (brain, plasma or saliva) were incubated with 200-μl of phosphate buffer containing different inhibitor concentrations. Iso-OMPA concentrations of 0.01 and 0.1 mM were incubated with brain and plasma samples, respectively (Maxwell et al., 1987; Mortensen et al., 1998). As determined in the current study, 0.1 mM iso-OMPA was incubated with saliva samples and a BW284C51 concentration of 0.01 mM was used for all assays. The total ChE activity was determined in the absence of ChE inhibitors. The reactions were terminated by adding an excess of buffer and ChE activity was measured as described below. The volume of excess buffer added was used to place the spectrophotometer detection (mOD min^{-1}) between 0.1 and 1.2 mOD min^{-1}. The final buffer dilution for brain and saliva was 1300- and 6-fold and for plasma was 40- and 180-fold dilution for BuChE and AChE, respectively.

2.5. Measurement of salivary BuChE activity profile and active site quantification

A titration of salivary BuChE enzyme with a known CPF-oxon concentration as described by Kardos and Sultatos (2000) was performed to determine the total enzyme active site concentration. Diluted saliva, 225 μl, was incubated with 25 μl phosphate buffer containing a range of CPF-oxon concentrations (0.1 pM–5 nM) in a shaker at room temperature up to 24 h without any inhibitors. Reactions were terminated by the addition of 0.75 ml phosphate buffer for each sample. Salivary BuChE activity was determined using a modified Ellman method (Ellman et al., 1961) in a 96-well automated microplate reader ELx808 equipped with a KC4 software package (Bio-Tek Instruments® Inc., Winooski, VT). For each well 250 μl of sample were added and 25 μl of DTNB and ATC or BTC were placed in each well giving a final concentration of 0.1 and 0.4 mM, respectively (Nostrandt et al., 1993; Mortensen et al., 1996). The control samples did not include CPF-oxon. The BuChE activity as described by the rate of ATC or BTC hydrolysis was monitored by following the absorbance profile at 405 nm over 30–40 min. The slope of the linear regression of that profile was used as a measure of the remaining enzyme activity (uninhibited BuChE).

The Kr (h^{-1}) and the total active site BuChE concentration were determined using a series of very low CPF-oxon concentrations (0.1–10 pM) until a CPF-oxon concentration equal to or less than the total BuChE concentration was found, as evidenced by achieving partial inhibition of the BuChE activity (Kardos and Sultatos, 2000). The Ki (nM^{-1} h^{-1}) of rat salivary BuChE was determined using higher CPF-oxon concentrations (0.25–5 nM) selected to give a maximum inhibition ranging from 10 to 90% over 7–30 min incubations. The Ki was also estimated by optimization of the pharmacodynamic model utilizing the estimated total BuChE active site concentration and Kr as described below.

2.6. Pharmacodynamic model development

The pharmacodynamic model for the description of the interaction of CPF-oxon with BuChE was developed in SIMUSOLV® (Mitchell and Gauthier Associates Inc., Concord, MA) based on the equation derived by Main (1964) as described by Kardos and Sultatos (2000). The differential equations describing the model are as follows:

$$\frac{\mathrm{dBuChE}}{\mathrm{d}t} = \mathrm{Ki} \times [\mathrm{CPFoxon}] \times [\mathrm{BuChE}] - \mathrm{Kr} \times [\mathrm{BuChE_{Phos}}] \quad (1)$$

$$[\mathrm{BuChE}] = [\mathrm{BuChE_t}] - [\mathrm{BuChE_{Phos}}] \quad (2)$$

$$[\mathrm{CPFoxon}] = [\mathrm{CPFoxon_t}] - [\mathrm{TCP}] \quad (3)$$

where $\mathrm{dBuChE}/\mathrm{d}t$ represents the change of the

uninhibited BuChE over time; CPF-oxon represents the chloropyrifos oxon concentration; $BuChE_t$ represents the total BuChE active site concentration; $BuChE_{Phos}$ represents the phosphorylated BuChE concentration; TCP represents the major metabolite of CPF-oxon (trichloropyridinol); Ki represents the apparent bimolecular inhibition rate constant of CPF-oxon towards BuChE; Kr represents the apparent reactivation rate constant of the phosphorylated BuChE; and $CPF\text{-}oxon_t$ represents the initial concentration of CPF-oxon.

2.7. Determination of the pharmacodynamic model parameters

To solve the model equations (Eqs. (1)–(3)), the initial BuChE active site concentration, and Kr values had to be determined experimentally and supplied to the model. The initial BuChE active site concentration was determined from the relationship between the CPF-oxon concentration and the maximum achieved BuChE inhibition, while the Kr was determined experimentally.

3. Results

3.1. Characterization of rat salivary ChE activity

Preliminary experiments indicated that the total salivary ChE activity was considerably less than that of plasma or brain homogenates since saliva dilution greater than 15-fold (vs. 180- to 1300-fold diluted plasma and brain, respectively) resulted in non-quantifiable ChE activity. In this regard, the rat salivary ChE active site concentration was estimated to be $\sim$12 000- and 1000-fold less than that of the brain and plasma ChE, respectively. Rat salivary ChE was found to be very stable when samples were stored at $-80\,^{\circ}C$ (up to 3 months). However, it was noted that in some samples the enzyme activity decreased particularly during longer incubation periods at room temp (>5 h) which necessitated the inclusion of parallel control samples for all longer incubation times. Repetitive daily saliva collection from the same rat (intra-individual variability) for several consecutive days

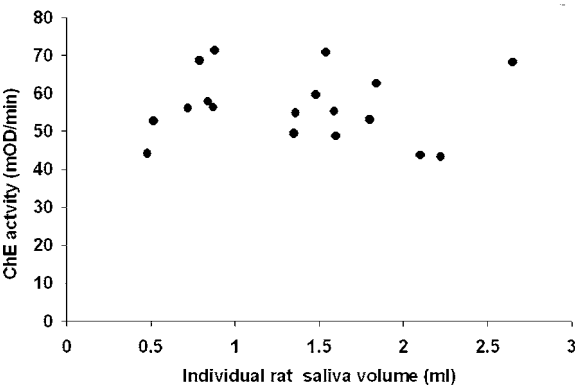

Fig. 1. In vitro determination of the individual adult male Sprague–Dawley rat salivary ChE activity described as the rate of ATC substrate hydrolysis (mOD min^{-1}) as a function of the total collected saliva volume over 30 min determined using a modified Ellman assay. Each circle represents an average of two determinations/rat (total 18 rats). The data suggests that ChE activity is independent of collected saliva volume.

(up to 4 days) did not result in appreciable differences in the total ChE activity (data not shown). As illustrated in Fig. 1, individual animal salivary ChE activity ranged from 40 to 70 mOD min^{-1} ($\sim$1.8-fold). In addition, although the volume of collected saliva ranged from 0.5 to 3.0 ml there were no observable changes with regards to total ChE activity as a function of volume suggesting that enzyme activity is independent of saliva volume or flow rate.

To characterize the total rat salivary ChE activity and the proportional contribution of AChE or BuChE towards that activity, the specific ChE activities of brain, plasma and saliva were determined using specific inhibitors for AChE (BW284C51) and BuChE (iso-OMPA) and compared with the control activity in the absence of inhibitors. The ChE activity was measured in the presence or absence of either inhibitor using ATC or BTC substrates and the results are presented in Fig. 2. Both AChE and BuChE have the capacity to hydrolyze ATC, but only BuChE hydrolyses BTC (Lassiter et al., 1998; Chuiko, 2000). A comparison of the in vitro ChE activity in brain, plasma, and saliva samples using ATC as the substrate with and without specific ChE inhibitors is presented in Fig. 2A. Incubation of rat brain homogenates with iso-OMPA using ATC had no

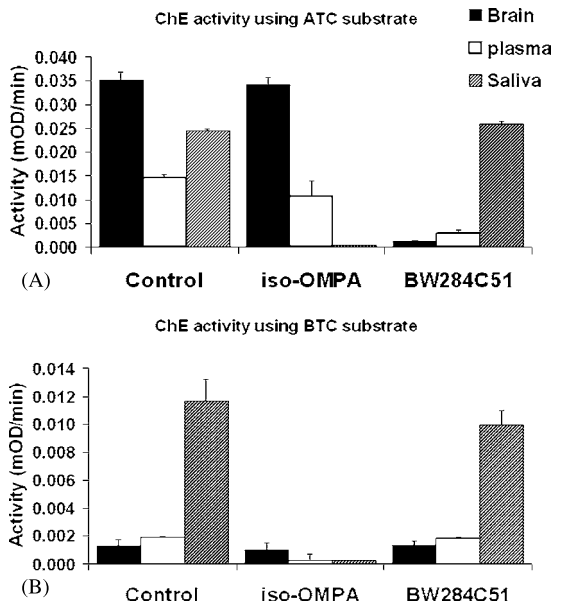

Fig. 2. In vitro determination of total ChE activity described as ATC (A) and BTC (B) hydrolysis rates in the controls and following iso-OMPA or BW284C51 incubation (15 min) with brain, plasma, and saliva samples obtained from naïve adult male Sprague–Dawley rats. Each bar represents the mean ± S.D. for three determinations. Sample dilution factors were 1300-fold for brain homogenate, 6-fold for saliva, and 40- and 180-fold dilution for plasma BuChE and AChE, respectively.

impact on the total brain ChE activity; whereas, incubation with BW284C51 resulted in a marked decrease (> 95%) of the total brain ChE activity. Plasma ChE activity was clearly inhibited by both iso-OMPA (~ 20% inhibition) and BW284C51 (~ 80% inhibition) since the total plasma ChE pool is comprised of both BuChE and AChE. In contrast to brain and plasma ChE activity, when saliva samples were incubated with iso-OMPA the inhibition of the total salivary ChE activity was nearly complete (> 95%), but the AChE specific inhibitor BW284C51 had virtually no impact on ChE activity. These results strongly indicate that more than 95% of the total rat salivary ChE activity is due to BuChE.

To further evaluate these results, the experiment was repeated using BTC as a substrate, since BTC is a relatively poor substrate for AChE and a good substrate for BuChE (Lassiter et al., 1998; Chuiko, 2000). A comparison of the in vitro ChE activity in brain, plasma, and saliva samples using BTC with and without the presence of the specific ChE inhibitors is presented in Fig. 2B. As expected, neither inhibitor appreciably impacted the total brain ChE activity compared with the control activity. However, it was noted that the total brain ChE activity towards BTC was 35-fold less than its activity towards ATC (compare Fig. 2A and B), which is primarily due to the poor BTC substrate affinity of brain AChE. Likewise, the total plasma ChE activity towards the BTC was also 7-fold less that the activity towards ATC, which is again due to the poor BTC substrate affinity of plasma AChE, and is also consistent with the insensitivity of the plasma ChE to BW284C51 inhibition using BTC. However, iso-OMPA resulted in nearly a complete loss of plasma ChE activity due to the high specificity of the inhibitor for BuChE and the poor affinity of the available plasma AChE for BTC. In addition, iso-OMPA markedly inhibited all salivary ChE activity when utilizing BTC (Fig. 2B) whereas; BW284C51 had no appreciable impact on total salivary ChE activity. These results further indicate that rat salivary ChE activity is primarily associated with BuChE.

3.2. Characterization of kinetic interaction of CPF-oxon with rat salivary BuChE

The dependence of the uninhibited BuChE enzyme activity on the CPF-oxon concentration was used to calculate the total BuChE active site concentration within the incubation as well as the Kr and the Ki of BuChE. As described by Kardos and Sultatos (2000), the kinetic scheme used in developing the pharmacodynamic model is based on the fact that, one molecule of CPF-oxon inhibits one molecule of BuChE by interacting with the serine hydroxyl group in the enzyme active site. Therefore, BuChE enzyme was titrated using a range of CPF-oxon concentrations (0.1–10 pM). A time-course illustrating the activity of salivary BuChE is presented in Fig. 3. Based on this time-course analysis, a maximum inhibition of BuChE was achieved by ~ 5 h post-incubation. Concentrations of 0.1 and 0.2 pM CPF-oxon inhibited ~ 40 and 85% of the total BuChE activity using ATC and ~ 50 and 75% inhibition using BTC. A final active site concentration was

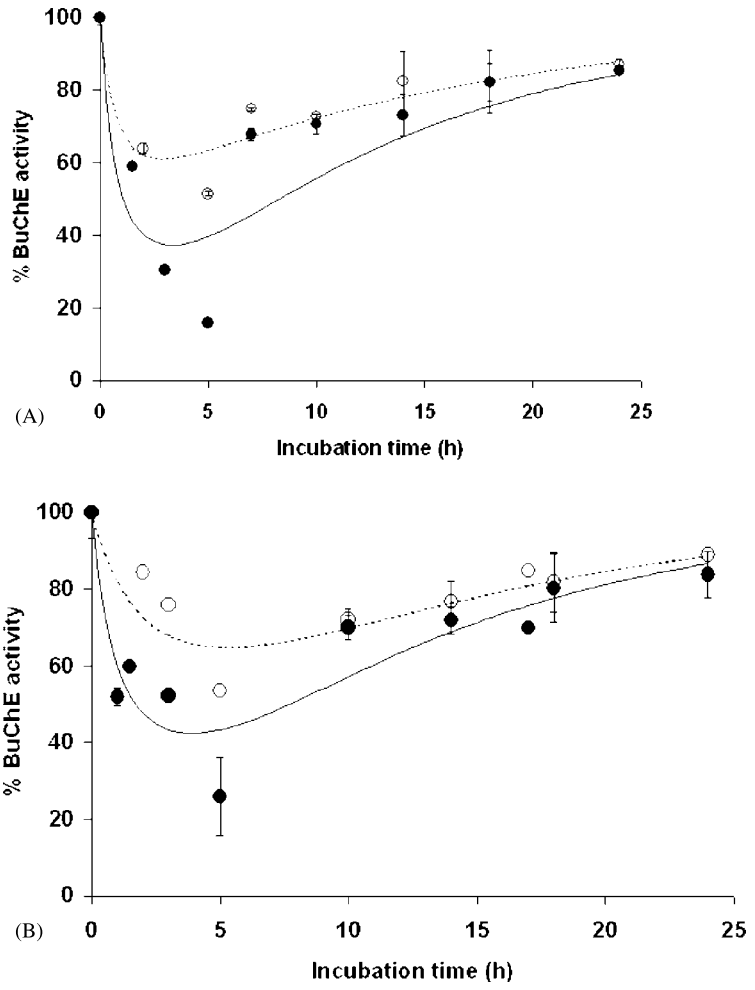

Fig. 3. Pharmacodynamic model simulation output against experimental data expressed as percentage of BuChE activity at CPF-oxon concentration of 0.1 pM (open circles and dashed lines) and 0.2 pM (filled circles and solid lines) incubated with saliva from naïve adult male Sprague–Dawley rats in the presence of ATC (A) and BTC (B) substrate, utilizing the experimentally determined Kr values (see Fig. 4). The maximum enzyme inhibition was achieved at 5 h. The model optimization resulted in a best-fit BuChE active site concentration of 0.213 and 0.236 pM/incubation for 0.1 and 0.2 pM CPF-oxon data respectively, in the presence of ATC (A) and 0.183 and 0.260 pM/incubation for 0.1 and 0.2 pM CPF-oxon data respectively, in the presence of BTC (B).

determined using the pharmacodynamic model through optimization of the model parameters against the experimental data. The pharmacodynamic model optimization yielded an average total salivary BuChE active site concentration of 0.223 ± 0.033 pM per incubation (see model fit Fig. 3 for the individual values). This estimation is consistent with the data, because BuChE reactivation and inhibition occurs simultaneously and 0.1 pM leads to ~ 40 and 50% inhibition of the total salivary ChE activity using ATC and BTC, respectively. Based upon the saliva dilution used in the current assay, this enzyme concentration represents an actual BuChE concentration of 1.20 ± 0.13 fmol ml^{-1} saliva.

The Kr was determined by transforming the percentage BuChE activity into a percentage BuChE inhibition where the slope of the log linear fit of the terminal portion of the curve equals the Kr (Levine and Murphy, 1977). The results of this

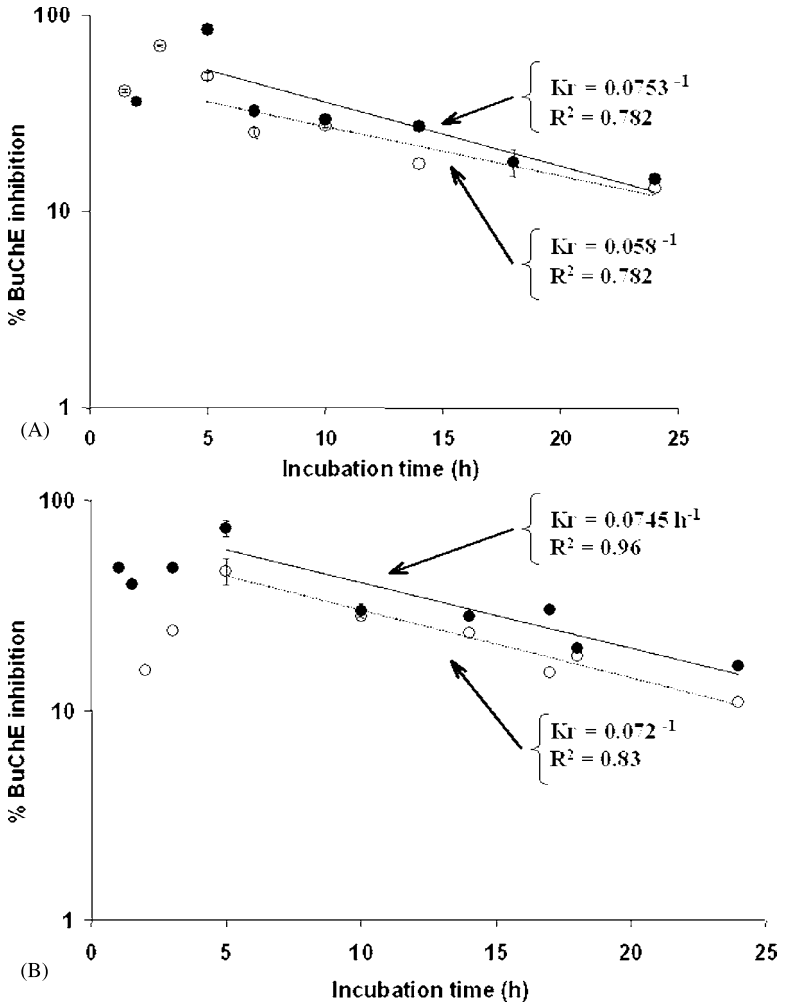

Fig. 4. In vitro determination of adult male Sprague–Dawley rat salivary BuChE spontaneous reactivation constant (Kr) following inhibition by 0.1 (open circles) and 0.2 pM (filled circles) CPF-oxon using ATC (A) and BTC (B) substrate. The data is expressed as percentage of BuChE inhibition and represents either an average of two determinations or, when appropriate, mean ± S.D. of three determinations. The lines (dashed and solid lines for 0.1 and 0.2 pM CPF-oxon concentration data, respectively) represent the best-fit for the terminal portion of the curve using linear regression.

analysis yielded an average Kr value using ATC and BTC of 0.070 ± 0.008 h^{-1}, the individual Kr values are shown in Fig. 4. The BuChE Ki was determined experimentally utilizing the approach of Main (1964). The rat saliva was incubated over time with a range of CPF-oxon concentrations (0.25–5 nM) and the percentage of BuChE activity, as described by ATC hydrolysis rate (mOD min^{-1}), was used for the Ki determination as illustrated in Fig. 5. The percentage of BuChE activity at different CPF-oxon concentrations was plotted against incubation time (Fig. 5A) and the slope of the linear regression of each data set was calculated. These slopes were plotted as a function of CPF-oxon concentration where 1/slope was plotted against 1/CPF-oxon concentration and the final Ki determination was based on the slope of the best-fit linear regression (Fig. 5B). Based on this analysis, the BuChE Ki was estimated to be 8.83 nM^{-1} h^{-1}. To further substantiate this

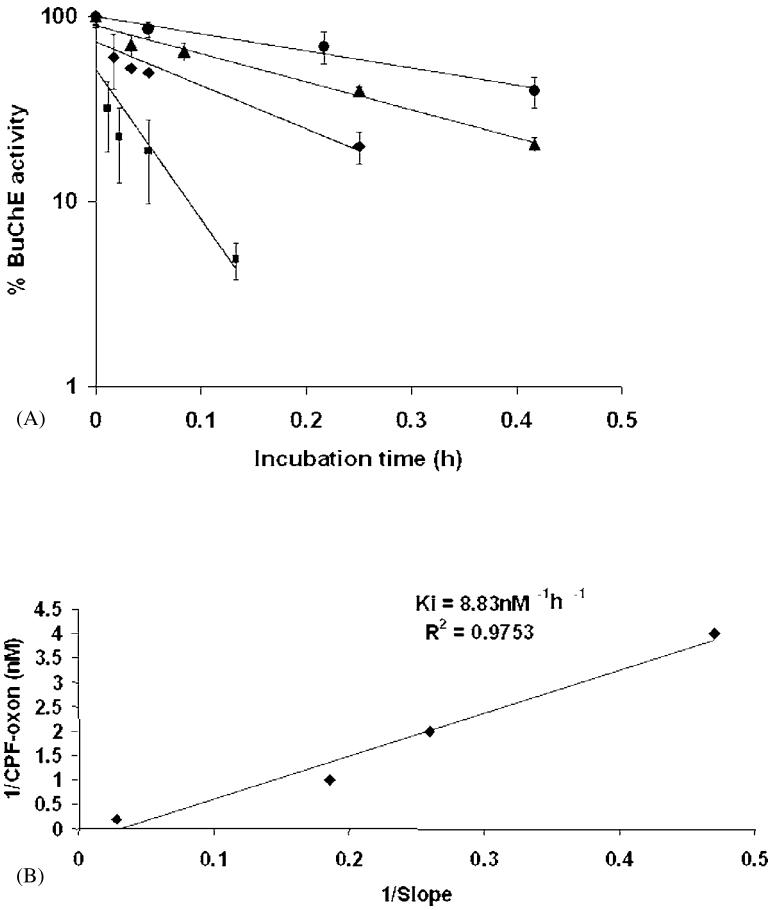

Fig. 5. (A) In vitro rat salivary BuChE activity (%BuChE activity) described as the rate of ATC substrate hydrolysis (mOD min^{-1}) as a function of CPF-oxon concentration for different incubation periods. Each data point represents the mean ± S.D. of triplicate samples of pooled saliva obtained from naïve adult male Sprague–Dawley rats. Circles represent CPF-oxon concentration of 0.25 nM; triangles, 0.5 nM; diamonds, 1.0 nM; and rectangles represent 5 nM. The lines represent the best fit from linear regression analysis for each data set. Slopes obtained from regression analysis showed a correlation > 0.95 and were used for the final Ki calculation (see Fig. 5B). (B) Final Ki determination plot. Each symbol represents a specific slope obtained from each data set at a given CPF-oxon concentration (see Fig. 5A). The straight line represents the best-fit by regression analysis and its slope equals the estimated Ki value.

estimate, the Ki was also determined using the pharmacodynamic model optimization subroutine of Simusolv by varying the Ki value while maintaining the parameter estimates for the total enzyme active site concentration and the Kr. The model was simultaneously fit to all experimental data sets shown in Fig. 5A and the results are presented in Fig. 6. Based upon this model optimization, a Ki value of 9.80 nM^{-1} h^{-1} provided a best fit to the data and was consistent with the 8.83 nM^{-1} h^{-1} determined by linear regression analysis (see Fig. 5).

4. Discussion

In the current study the optimal experimental requirements to characterize rat salivary ChE activity were determined using a modified Ellman assay in conjunction with a pharmacodynamic model. Although numerous reports have been published on the estimation of plasma and RBC ChE activity following OP insecticide exposure, data on human or rat salivary ChE is limited (Borzelleca and Skalsky, 1980; Ryhanen, 1983; Ryhanen et al., 1983; Yamalik et al., 1991).

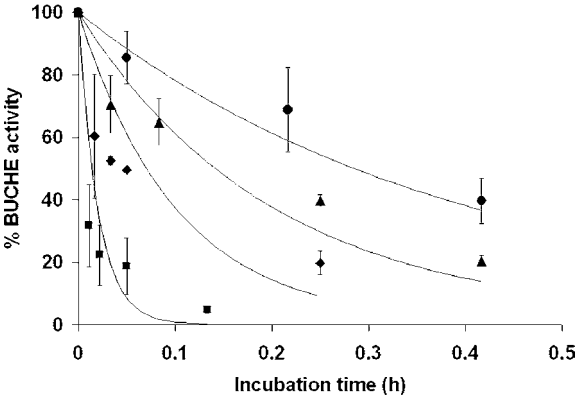

Fig. 6. Ki calculation using the pharmacodynamic model optimizations against the experimental data. The data represent percentage of saliva BuChE activity described as the rate of ATC substrate hydrolysis (mOD min^{-1}) as a function of CPF-oxon concentration for different incubation periods. Each data point represents the mean of triplicate samples of pooled saliva obtained from naïve adult male Sprague–Dawley rats. Circles represent CPF-oxon concentration of 0.25 nM; triangles, 0.5 nM; diamonds, 1.0 nM; and rectangles represent 5 nM. Each line represents the model simulation for a particular data set.

Salivary biomonitoring has recently been explored as a practical method for examination of pesticide exposure through the measurement of the pesticides or their metabolites in saliva (Nigg and Wade, 1992; Lu et al., 1997). However, we are aware only of a single study concerning the use of salivary ChE for monitoring OP insecticide exposure (Borzelleca and Skalsky, 1980). In order to use salivary ChE as a biological monitor for OP insecticide exposure, the total enzyme activity and kinetic parameters need to be characterized. In the present study, total adult male Sprague–Dawley rat salivary ChE activity, the proportional contribution of AChE and BuChE towards the total salivary ChE activity, and the salivary BuChE kinetic parameters were determined. Hansen et al. (1992) noted marked loss of salivary ChE activity at 37 °C in human saliva, which is comparable to the results, reported here. In the current study, salivary ChE activity was stable when measured on a repetitive daily basis (up to 4 days), however, some degree of inter-individual variability was noticed (Fig. 1) which is also consistent with Hansen et al. (1992) who reported less intra-individual than inter-individual variability in human salivary ChE activity.

Although the salivary BuChE active site concentration was ~12 000- and 1000-fold less than that of the brain and plasma, respectively it could be reliably measured using a modified Ellman et al. (1961) assay and its kinetic profile was successfully followed over time after in vitro incubation with various CPF-oxon concentrations. Ryhanen et al. (1983) estimated a total human salivary ChE activity to be 1500-fold lower than human plasma ChE activity, which is consistent with the observed difference in the rat determined in the current study.

Incubation of 0.1 mM BW284C51 as described by Lassiter et al. (1998) resulted in excessive loss of plasma BuChE as evidenced by a decreased BTC hydrolysis rate. Therefore, a concentration of 0.01 mM BW284C51 was used for all samples to avoid non specific inhibition of BuChE. Characterization of rat salivary and brain ChE activity in the presence of specific AChE and BuChE inhibitors indicated that >95% of the total ChE activity in whole saliva was associated with BuChE, whereas >95% of brain ChE was associated with AChE. As illustrated in Fig. 2A plasma ChE was decreased by ~20 and 80% following incubation with either iso-OMPA or BW284C51, respectively, compared with the controls. However, it should be noted that the substrate used in this experiment was ATC which has been shown to be hydrolyzed by AChE with a hydrolysis rate that is four times (140 000 vs. 40 000 s^{-1}, respectively) higher than its BuChE hydrolysis rate (Taylor, et al., 1995). Based on this relationship rat plasma ChE activity determined in the present study is equally distributed between AChE and BuChE (50% AChE and 50% BuChE). Hence, the estimated values for brain and plasma ChE activity using specific inhibitors and substrates are very consistent with the activity reported in the literature (Traina and Serpietri, 1984; Maxwell et al., 1987; Sultatos, 1994; Milatovic and Dettbarn, 1996). Although the characterization of rodent salivary ChE has not been previously reported, the presence of BuChE in human saliva has been previously demonstrated (Ryhanen, 1983; Ryhanen et al., 1983; Yamalik et al., 1991). Ryhanen et al. (1983)

reported that in human saliva, BuChE activity constituted 70–90% of the whole ChE activity. In another study Ueda and Yamaguchi (1976) reported that ChE in whole saliva was comprised of both AChE and BuChE, with BuChE being the major ChE form. Therefore, since both rodent and human saliva ChE is predominantly BuChE, the esterase inhibition kinetics observed in the current study suggests that human saliva esterase may demonstrate a similar kinetic response following exposure to CPF-oxon. Although this suggests that human salivary ChE may represent a sensitive biomarker for assessing OP insecticide exposure, additional in vitro studies using human derived saliva are needed for validation.

The utility of salivary ChE activity as a biological monitor for OP insecticide exposure will be of greater value if it reflects and correlates with systemic levels of ChE activity (i.e. RBC or plasma ChE). Ryhanen (1983) reported that salivary ChE activity in humans was unaffected by varying salivary flow-rate (0.2–3.1 ml min^{-1}). In addition, Lu et al. (1997) and Borzelleca and Skalsky (1980) suggested that the atrazine and carbaryl concentrations could reasonably be measured in saliva, since the saliva/plasma ratios of atrazine and carbaryl levels were also unaffected by salivary flow rate. In the current study, salivary ChE activity likewise was independent of the variation in total collected saliva volume (Fig. 1). Therefore, rat salivary ChE activity determined in this study appears to be well suited for biomonitoring exposure to CPF or other similar OP insecticides. Borzelleca and Skalsky (1980) reported that rat salivary ChE inhibition correlated with the plasma and RBC ChE activity following carbaryl exposure, however, no dose–response relationship was observed and no attempt was made to characterize the proportional contribution of AChE and BuChE toward the total ChE activity. Further evaluation of their data suggests that the measured ChE activity may have achieved its maximum inhibition at the lowest dose level, which would make it difficult to characterize a dose–response relationship. However, of particular interest was the observation that the salivary ChE inhibition response was comparable or greater than that obtained with the plasma and RBC, which would be expected with a pure source of BuChE.

With respect to the determination of BuChE kinetic parameters, the current study demonstrated that rat saliva is a good source of relatively pure BuChE and is a useful matrix to characterize the biological interaction of BuChE with CPF-oxon and other OP insecticides. The average Kr value of the inhibited BuChE was 0.070 ± 0.008 h^{-1}. Although there was no previously reported Kr value for inhibited rat BuChE by CPF-oxon, this Kr value is comparable with the value reported by Kardos and Sultatos (2000) for the inhibition of rat brain AChE by paraoxon, another phosphothioate insecticide. It is of interest to note that several previously published PBPK/PD models for the OP insecticides assumed that BuChE reactivation is similar to that of AChE (Gearhart et al., 1990, 1994; Timchalk et al., 2002b).

Spontaneous recovery plays an important role during assessment of ChE response following Op exposure. Pope et al. (1991) reported that among different OP insecticides, ChE recovery in vivo is probably related to differences in absorption, biotransformation, and most importantly spontaneous ChE reactivation. Since both human and rat saliva ChE activities are mainly due to BuChE, the estimated Kr of rat salivary BuChE in the current study suggests a similar estimate for human BuChE. The pharmacodynamic model was used to estimate the BuChE Ki to be 9.80 nM^{-1} h^{-1} (Fig. 6), which is consistent with 8.83 nM^{-1} h^{-1} determined using the approach of Main (1964); see Fig. 5, but is lower than the 80 nM^{-1} h^{-1} reported by Amitai et al. (1998) for mouse recombinant BuChE. However, the Ki determined by Amitai et al. was based on a CPF-oxon interaction with a purified BuChE, a less complex matrix compared with the saliva used in the current study. In addition, the estimated Ki in the current study is consistent with an optimized estimate that was used by Timchalk et al. (2002b) that adequately fit in vivo BuChE inhibition kinetics in both rats and humans.

Whereas pharmacokinetic models seek to describe the disposition of chemicals, pharmacodynamic models seek to quantify the biological

responses to chemicals (Mager and Jusko, 2001). When extensive mechanistic data are available for chemicals, pharmacodynamic models can be constructed that describe detailed events that lead to a measurable biological response. For example, Maxwell et al. (1988) developed an approach for modeling the inhibition of AChE by cholinesterase inhibitors that included all significant events, known at that time, in the interaction of OP insecticide with AChE. Similarly in the current study, a pharmacodynamic model that describes the in vitro kinetics of BuChE and CPF-oxon interaction was developed and used to estimate the BuChE active site concentration and activity profile as well as the Ki estimation (see Figs. 3 and 6). It should be emphasized that, in those experiments where sample availability is limited, modeling of the experimental data represents a very effective tool for further prediction of biological events as evidenced by the use of the current model for predicting BuChE active site concentration and Ki.

Although the physiological role of BuChE in mammalian tissues has not yet been fully determined, several studies suggest that BuChE inhibition protects against the AChE inhibition by OP insecticides (Junge and Krisch, 1975; Fonnum et al., 1985; Maxwell et al., 1987; Chambers et al., 1990; Chanda et al., 1997; Yang and Dettbarn, 1998). The estimated rat salivary BuChE Ki in the current study was almost 45-fold (~ 9 vs. ~ 0.2 $nM^{-1}\ h^{-1}$) higher than the Ki value reported for AChE inhibition by CPF-oxon (Carr and Chambers, 1996). The higher BuChE Ki may lead to a conceivable assumption that BuChE serves as an efficient endogenous scavenger of CPF-oxon thereby, protecting against CPF-oxon toxicity. In this regard, Amitai et al. (1998) proposed that BuChE could be given intravenously as an exogenous scavenger against AChE inhibition in case of severe OP insecticide poisoning.

In summary, these study results clearly characterized the rat salivary ChE activity and the contributions of AChE and BuChE towards the total activity as well as the kinetic parameters describing the interaction of CPF-oxon with BuChE. The results also demonstrated the value of using rat saliva as a relatively pure BuChE source for the estimation of the kinetic parameters for BuChE interaction with CPF-oxon as well as similar OP insecticides. Although future studies are needed to assess the ChE response in human saliva and to further evaluate the sensitivity of saliva ChE biomonitoring following in vivo CPF exposure in animals, the overall results of the current study do support the potential utility of saliva ChE for biomonitoring exposure to CPF and other important OP insecticides.

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